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(54) **Title:** IMPROVED IMMUNOGENICITY USING A COMBINATION OF DNA AND VACCINIA VIRUS VECTOR VACCINES

(57) **Abstract:** This invention relates to improved methods of inducing an immune response for the prevention or treatment of HIV-1 infection by using a nucleic acid vaccine in conjunction with a recombinant viral vaccine, e.g., a poxvirus vaccine, to potentiate and broaden the immune response. The present invention further provides a particularly effective vaccine regimen comprising a DNA vaccine used in combination with a poxvirus virus, especially NYVAC or ALVAC.

IMPROVED IMMUNOGENICITY USING A COMBINATION OF DNA AND VACCINIA VIRUS VECTOR VACCINES

CROSS-REFERENCE TO RELATED APPLICATIONS

The present application claims the benefit of priority of U.S. Provisional Application 60/200,444 filed April 28, 2000, which is herein incorporated by reference.

STATEMENT AS TO RIGHTS TO INVENTIONS MADE UNDER FEDERALLY SPONSORED RESEARCH AND DEVELOPMENT

[Not Applicable]

FIELD OF THE INVENTION

This invention relates to improved methods of inducing an immune response for the prevention or treatment of human immunodeficiency virus-1 (HIV-1) infection by using a nucleic acid vaccine in conjunction with a recombinant viral vaccine, *e.g.*, a poxvirus vaccine, to potentiate and broaden the immune response. The present invention further provides a particularly effective vaccine regimen comprising a DNA vaccine used in combination with a recombinant poxvirus virus, especially NYVAC or ALVAC.

SUMMARY OF THE INVENTION

The present invention is directed to a method of stimulating an immune response in a human at risk for infection, or infected with, an HIV-1 retrovirus. The method comprises administering a first vaccine, frequently a nucleic acid vaccine, which enters the cells and intracellularly produces HIV-specific peptides for presentation on the cell's MHC class I molecules in an amount sufficient to stimulate a CD8⁺ immune response. The first vaccine may be used in combination with a second vaccine comprising another modality, *e.g.*, a recombinant pox virus vector. The use of the combination of vaccines potentiates the immune response relative to the use of either of the vaccines alone.

The vaccine combination may be administered prophylactically to individuals at risk of HIV infection. To prevent infection, individuals who are non vaccinia-naïve may particularly benefit. Alternatively, patients already infected with HIV may receive the vaccine regimen therapeutically. Patients who are candidates for treatment with the vaccine regimen of the invention include those who have a viral load of less than 10,000 viral copies per ml of plasma and a CD4⁺ cell count of above 500 cells/ml. Other patients include those who have been treated with one or more anti-viral agents.

The method frequently employs a nucleic acid vaccine that is a DNA vaccine in combination with an attenuated recombinant virus. A preferred virus is an attenuated pox virus, particularly NYVAC and ALVAC, attenuated vaccinia and canarypox viruses respectively. The DNA vaccine and/or the recombinant virus vaccine may be administered one or more times. The DNA vaccine is preferably administered prior to administration of the recombinant virus vaccine, frequently in multiple doses. In one embodiment, a DNA vaccine encoding various HIV antigens, or epitopes derived from the antigens, is administered multiple times prior to administration of a NYVAC-HIV vaccine.

The vaccine may also comprise interleukin-2 (IL-2) or CD40 ligand in an amount that is sufficient to further potentiate the CD8⁺ and CD4⁺ T-cell responses.

BRIEF DESCRIPTION OF THE FIGURES

Figure 1: Figure 1 shows the study design evaluating a combination DNA vaccine/NYVAC vaccine in *Rhesus macaques*. Three groups of 8 rhesus macaques each were included. The animals were immunized four times with either mock NYVAC (group A) or NYVAC-SIV-*gag-pol-env* (group B), or three times with DNA-SIV-*gag-env* followed by two immunizations with NYVAC-SIV-*gag-pol-env* at the times indicated.

Figure 2: Figure 2 shows lymphoproliferative responses to gp120 (upper panel) and p27 antigens (lower panel) in the three groups inoculated in accordance with the study design set forth in Figure 1.

Figure 3: Figure 3 shows the frequency of Gag181-specific CD8⁺ T-cells in peripheral blood monocytes (PBMC) of vaccinated macaques as measured by IFN- γ ELISPOT assay at the times indicated. "S.F.C." indicates spot-forming cells/per million; "N.D." indicates not done. An asterisk above the bar marks the values obtained by assaying frozen cells.

Figure 4: Figure 4 shows Gag181-specific tetramer staining of fresh PBMC at week 53 and 76. The cells depicted in the Figure were first gated for CD3⁺ population.

Figure 5: Figure 5 shows the T-cell responses to various SIV epitopes measured using ELISPOT and ⁵¹Cr-release assays.

Figure 6: Figure 6 shows the average group viremia during the first 28 days following intrarectal challenge with SIVmac251. Points represent group means with standard error indicated by the bar.

Figure 7: Figure 7 shows the average value of Gag181 tetramer-specific staining in all MAMU-A*01-positive animals in each group after challenge. Points represent the mean of the percentage of Gag181 tetramer-positive cells of the total CD3⁺CD8⁺ T-cell population with standard errors indicated by bars.

Figure 8: Figure 8 shows the average group viremia in MAMU A*01-positive and MAMU A*01-negative animals of control and DNA/NYVAC-SIV vaccinated (group C) animals. In the control group, the results obtained in two studies were combined. Points represent group means with standard errors indicated by the bars.

Definitions

"Attenuated recombinant virus" refers to a virus that has been genetically altered by modern molecular biological methods, *e.g.* restriction endonuclease and ligase treatment, and rendered less virulent than wild type, typically by deletion of specific genes or by serial passage in a non-natural host cell line or at cold temperatures.

"Efficient CD8⁺ response" is referred to as the ability of cytotoxic CD8⁺ T-cells to recognize and kill cells expressing foreign peptides in the context of a major histocompatibility complex (MHC) class I molecule.

"Nonstructural viral proteins" are those proteins that are needed for viral production but are not necessarily found as components of the viral particle. They include DNA binding proteins and enzymes that are encoded by viral genes but which are not present in the virions. Proteins are meant to include both the intact proteins and fragments of the proteins or peptides which are recognized by the immune cell as epitopes of the native protein.

A "nucleic acid vaccine" or "naked DNA vaccine" refers to a vaccine that includes one or more expression vectors that encodes B-cell and/or T-cell epitopes and provides an immunoprotective response in the person being vaccinated. As used herein, the term does not include a recombinant pox viral vaccine.

"Plasma" refers to the fraction of whole blood resulting from low speed centrifugation of EDTA- or heparin- treated blood.

"Pox viruses" are large, enveloped viruses with double-stranded DNA that is covalently closed at the ends. Pox viruses replicate entirely in the cytoplasm, establishing discrete centers of viral synthesis. Their use as vaccines has been known since the early 1980's (*see, e.g. Panicali, D. et al. "Construction of live vaccines by using genetically engineered pox viruses: biological activity of recombinant vaccinia virus expressing influenza virus hemagglutinin", Proc. Natl. Acad. Sci. USA 80:5364-5368, 1983*).

"Potentiating" or "enhancing" an immune response means increasing the magnitude and/or the breadth of the immune response, *i.e.*, the number of cells induced by a particular epitope may be increased and/or the numbers of epitopes that are recognized may be increased ("breadth"). A 5-fold, often 10-fold or greater, enhancement in both CD8⁺ and CD4⁺ T-cell responses is obtained with administration of a combination of nucleic acid/recombinant virus vaccines compared to administration of either vaccine alone.

A "retrovirus" is a virus containing an RNA genome and an enzyme, reverse transcriptase, which is an RNA-dependent DNA polymerase that uses an RNA molecule as a template for the synthesis of a complementary DNA strand. The DNA form of a retrovirus commonly integrates into the host-cell chromosomes and remains part of the host cell genome for the rest of the cell's life.

“Structural viral proteins” are those proteins that are physically present in the virus. They include the capsid proteins and enzymes that are loaded into the capsid with the genetic material. Because these proteins are exposed to the immune system in high concentrations, they are considered to be the proteins most likely to provide an antigenic and immunogenic response. Proteins are meant to include both the intact proteins and fragments of the proteins or peptides which are recognized by the immune cell as epitopes of the native protein.

“Viral load” is the amount of virus present in the blood of a patient. Viral load is also referred to as viral titer or viremia. Viral load can be measured in variety of standard ways. In preferred embodiments, the DNA/recombinant virus prime boost protocol of the invention controls viremia and leads to a greater reduction in viral load than that obtained when either vaccine is used alone.

DETAILED DESCRIPTION

Introduction

Recombinant pox viruses vaccines, *e.g.*, NYVAC- and ALVAC-based vaccines for HIV-1 have been tested in preclinical trials using either HIV-2 or SIV *Gag*, *Pol*, and *Env* genes in macaques (*see, e.g.*, Benson *et al.*, *J. Virol.* 72:4170-4182, 1998; Abimiku *et al.*, *J. Acquir. Immune Defic. Synd. Hum. Retrovirol.* 15:S78-S85, 1997; Myagkikh *et al.*, *AIDS Res. Hum. Retroviruses* 12:985-991, 1996; and Hel *et al.*, *Nat. Med.* 16:1140-1146, 2000). Results from these early studies indicated that, while these vaccines do not protect from infection, they significantly reduce the viral replication within a few weeks from exposure in approximately 50% of the animals. In the case of NYVAC-SIV vaccination, the regimen changed the natural course of SIV₂₅₁ infection.

In the macaque animal model, the addition of monomeric gp120 protein administered as a boost in conjunction with ALVAC-SIV_{gpe} did not appear to improve the level of protection. (*see, e.g.*, Pal *et al.*, Abstract for “HIV/AIDS Vaccine Development Workshop,” Paris, France, May 5-6, 2000). These studies also suggested that more than three immunizations with NYVAC-SIV/ALVAC-SIV may not further increase the pool of memory cells, and that the vector immunity against vaccinia protein may blunt the response to SIV antigens.

Various other prime boost immunization strategies against HIV have also been proposed (*see, e.g., Barnett et al., AIDS Res. and Human Retroviruses* Volume 14, Supplement 3, 1998, pp. S-299-S-309 and Girard *et al., C R Acad. Sci III* 322:959-966, 1999 for reviews). DNA immunization, when used in a boosting
5 protocol with modified vaccinia virus Ankara (MVA) or with a recombinant fowl pox virus (rFPV) in the macaque model, has been shown to induce CTL responses and antibody responses (*see, e.g., Hanke et al., J. Virol.* 73:7524-7532, 1999; Hanke *et al., Immunol. Letters* 66:177-181; Robinson *et al., Nat. Med.* 5:526-534, 1999), but no protection from a viral challenge was achieved in the immunized animals. DNA
10 immunization followed by administration of another highly attenuated poxvirus has also been tested for the ability to elicit IgG responses, but the interpretation of the results is hampered by the fact that serial challenges were performed (*see, e.g., Fuller et al., Vaccine* 15:924-926, 1997; Barnett *et al., supra*). In contrast, in a murine model of malaria, DNA vaccination used in conjunction with a recombinant vaccinia
15 virus was promising in protecting from malaria infection (*see, e.g., Sedegah et al., Proc. Natl. Acad. Sci. USA* 95:7648-7653, 1998; Schneider *et al., Nat. Med.* 4:397-402, 1998).

The present invention provides for enhanced immunogenicity of a recombinant poxvirus-based vaccine by administering a nucleic acid, *e.g.,* a DNA
20 vaccine, to stimulate an immune response to the HIV antigens provided in the poxvirus vaccine, and thereby increase the ability of the recombinant pox virus, *e.g.,* NYVAC or ALVAC, to expand a population of immune cells.

Individuals who are treated with the vaccine regimen may be at risk for infection with the virus or may have already been infected.

Vaccines of use in this invention

Vaccines useful for the induction of CD8⁺ T-cell responses comprise nucleic acid vaccines (preferably delivered as a DNA vaccine) and recombinant pox virus vaccines that provide for the intracellular production of viral-specific peptide
30 epitopes that are presented on MHC Class I molecules and subsequently induce an immunoprotective cytotoxic T lymphocyte (CTL) response.

The invention contemplates single or multiple administrations of the nucleic acid vaccine in combination with one or more administrations of the recombinant virus vaccine. This vaccination regimen may be complemented with

administration of recombinant protein vaccines, or may be used with additional vaccine vehicles. Preferably, administration of the nucleic acid vaccine precedes administration of the recombinant virus vaccine.

In preferred embodiments, the DNA/recombinant virus prime boost
5 protocol controls viremia and reduces viral load as well as potentiating a CD8⁺ response.

Attenuated recombinant viral vaccines

Attenuated recombinant poxviruses that express retrovirus-specific epitopes
10 are of use in this invention. Attenuated viruses are modified from their wildtype virulent form to be either symptomless or weakened when infecting humans. Typically, the genome of the virus is defective in respect of a gene essential for the efficient production or essential for the production of infectious virus. The mutant virus acts as a vector for an immunogenic retroviral protein by virtue of the virus encoding foreign DNA. This provokes or stimulates
15 a cell-mediated CD8⁺ response.

The virus is then introduced into a human vaccinee by standard methods for vaccination of live vaccines. A live vaccine of the invention can be administered at, for example, about 10^4 - 10^8 organisms/dose, or 10^6 to 10^9 pfu per dose. Actual dosages of such a vaccine can be readily determined by one of ordinary skill in the field of vaccine
20 technology.

The poxviruses are of preferred use in this invention. There are a variety of attenuated poxviruses that are available for use as a vaccine against HIV. These include attenuated vaccinia virus, cowpox virus and canarypox virus. In brief, the basic technique of inserting foreign genes into live infectious poxvirus involves a recombination between pox
25 DNA sequences flanking a foreign genetic element in a donor plasmid and homologous sequences present in the rescuing poxvirus as described in Piccini *et al.*, Methods in Enzymology 153, 545-563 (1987). More specifically, the recombinant poxviruses are constructed in two steps known in the art and analogous to the methods for creating synthetic recombinants of poxviruses such as the vaccinia virus and avipox virus described
30 in U.S. Patent Nos. 4,769,330, 4,722,848, 4,603,112, 5,110,587, and 5,174,993, the disclosures of which are incorporated herein by reference.

First, the DNA gene sequence encoding an antigenic sequence such as a known T-cell epitope is selected to be inserted into the virus and is placed into an *E. coli* plasmid construct into which DNA homologous to a section of DNA of the poxvirus has

been inserted. Separately, the DNA gene sequence to be inserted is ligated to a promoter. The promoter-gene linkage is positioned in the plasmid construct so that the promoter-gene linkage is flanked on both ends by DNA homologous to a DNA sequence flanking a region of pox DNA containing a nonessential locus. The resulting plasmid construct is then
5 amplified by growth within *E. coli* bacteria.

Second, the isolated plasmid containing the DNA gene sequence to be inserted is transfected into a cell culture, *e.g.* chick embryo fibroblasts, along with the poxvirus. Recombination between homologous pox DNA in the plasmid and the viral genome respectively gives a poxvirus modified by the presence, in a nonessential region of
10 its genome, of foreign DNA sequences.

Attenuated recombinant pox viruses are a preferred vaccine. A detailed review of this technology is found in US Patent No. 5,863,542, which is incorporated by reference herein. These viruses are modified recombinant viruses having inactivated virus-encoded genetic functions so that the recombinant virus has attenuated virulence and
15 enhanced safety. The functions can be non-essential, or associated with virulence. The poxvirus is generally a vaccinia virus or an avipox virus, such as fowlpox virus and canarypox virus. The viruses are generated using the general strategy outlined above and in US Patent no. 5,863,542.

Representative examples of recombinant pox viruses include ALVAC,
20 TROVAC, NYVAC, and vCP205 (ALVAC-MN120TMG). These viruses were deposited under the terms of the Budapest Treaty with the American Type Culture Collection (ATCC), 12301 Parklawn Drive, Rockville, Md., 20852, USA: NYVAC under ATCC accession number VR-2559 on Mar. 6, 1997; vCP205 (ALVAC-MN120TMG) under ATCC accession number VR-2557 on Mar. 6, 1997; TROVAC under ATCC accession number VR-2553 on
25 Feb. 6, 1997 and, ALVAC under ATCC accession number VR-2547 on Nov. 14, 1996.

NYVAC is a genetically engineered vaccinia virus strain generated by the specific deletion of eighteen open reading frames encoding gene products associated with virulence and host range. NYVAC is highly attenuated by a number of criteria including: i) decreased virulence after intracerebral inoculation in newborn mice, ii) inocuity in
30 genetically (nu^+/nu^+) or chemically (cyclophosphamide) immunocompromised mice, iii) failure to cause disseminated infection in immunocompromised mice, iv) lack of significant induration and ulceration on rabbit skin, v) rapid clearance from the site of inoculation, and vi) greatly reduced replication competency on a number of tissue culture cell lines including those of human origin.

TROVAC refers to an attenuated fowlpox that was a plaque-cloned isolate derived from the FP-1 vaccine strain of fowlpoxvirus which is licensed for vaccination of 1 day old chicks.

ALVAC is an attenuated canarypox virus-based vector that was a plaque-cloned derivative of the licensed canarypox vaccine, Kanapox (Tartaglia *et al.*, 1992). ALVAC has some general properties which are the same as some general properties of Kanapox. ALVAC-based recombinant viruses expressing extrinsic immunogens have also been demonstrated efficacious as vaccine vectors. This avipox vector is restricted to avian species for productive replication. On human cell cultures, canarypox virus replication is aborted early in the viral replication cycle prior to viral DNA synthesis. Nevertheless, when engineered to express extrinsic immunogens, authentic expression and processing is observed *in vitro* in mammalian cells and inoculation into numerous mammalian species induces antibody and cellular immune responses to the extrinsic immunogen and provides protection against challenge with the cognate pathogen.

NYVAC, ALVAC and TROVAC have also been recognized as unique among all poxviruses in that the National Institutes of Health ("NIH")(U.S. Public Health Service), Recombinant DNA Advisory Committee, which issues guidelines for the physical containment of genetic material such as viruses and vectors, *i.e.*, guidelines for safety procedures for the use of such viruses and vectors which are based upon the pathogenicity of the particular virus or vector, granted a reduction in physical containment level: from BSL2 to BSL1. No other poxvirus has a BSL1 physical containment level. Even the Copenhagen strain of vaccinia virus-the common smallpox vaccine-has a higher physical containment level; namely, BSL2. Accordingly, the art has recognized that NYVAC, ALVAC and TROVAC have a lower pathogenicity than any other poxvirus.

Another attenuated poxvirus of preferred use for this invention is Modified Vaccinia virus Ankara (MVA), which acquired defects in its replication ability in humans as well as most mammalian cells following over 500 serial passages in chicken fibroblasts (*see, e.g., Mayr et al., Infection* 3:6-14 (1975); Carrol, M. and Moss, B. *Virology* 238:198-211 (1997)). MVA retains its original immunogenicity and its variola-protective effect and no longer has any virulence and contagiousness for animals and humans. As in the case of NYVAC or ALVAC, expression of recombinant protein occurs during an abortive infection of human cells, thus providing a safe, yet effective, delivery system for foreign antigens.

The HIV antigen-encoding DNA for insertion into these vectors are any that are known to be effective antigens for protection against a retrovirus. These can include

both structural and non-structural proteins. The envelope, polymerase, gag, and protease are preferred proteins or sources of epitopes, but other proteins or epitopes can also be employed including those proteins encoded by non-structural genes, *e.g.*, *rev*, *tat*, *nef*, *vif*, and *vpr*. For HIV, nucleic acids that can be inserted into the viral vector includes, but are not limited to, nucleic acid that can code for at least one of: HIV1gag(+ pro)(IIIB), gp120(MN)(+ transmembrane), nef(BRU)CTL, pol(IIIB)CTL, ELDKWA or LDKW epitopes, preferably HIV1gag(+ pro)(IIIB), gp120(MN) (+ transmembrane), two (2) nef(BRU)CTL and three (3) pol(IIIB)CTL epitopes; or two ELDKWA in gp120 V3 or another region or in gp160. The two (2) nef(BRU)CTL and three (3) pol(IIIB)CTL epitopes are preferably CTL1, CTL2, pol1, pol2 and pol3. In the above listing, the viral strains from which the antigens are derived are noted parenthetically.

Nucleic acid vaccines

The vaccine combination of the invention typically includes as one of the vaccines a nucleic acid vaccine, preferably DNA. Nucleic acid vaccines as defined herein, typically plasmid expression vectors, are not encapsidated in a viral particle. The nucleic acid vaccine is directly introduced into the cells of the individual receiving the vaccine regimen. This approach is described, for instance, in Wolff *et. al.*, *Science* 247:1465 (1990) as well as U.S. Patent Nos. 5,580,859; 5,589,466; 5,804,566; 5,739,118; 5,736,524; 5,679,647; and WO 98/04720. Examples of DNA-based delivery technologies include, "naked DNA", facilitated (bupivacaine, polymers, peptide-mediated) delivery, and cationic lipid complexes or liposomes. The nucleic acids can be administered using ballistic delivery as described, for instance, in U.S. Patent No. 5,204,253 or pressure (*see, e.g.*, U.S. Patent No. 5,922,687). Using this technique, particles comprised solely of DNA are administered, or in an alternative embodiment, the DNA can be adhered to particles, such as gold particles, for administration.

As is well known in the art, a large number of factors can influence the efficiency of expression of antigen genes and/or the immunogenicity of DNA vaccines. Examples of such factors include the reproducibility of inoculation, construction of the plasmid vector, choice of the promoter used to drive antigen gene expression and stability of the inserted gene in the plasmid.

Any of the conventional vectors used for expression in eukaryotic cells may be used for directly introducing DNA into tissue. Expression vectors containing

regulatory elements from eukaryotic viruses are typically used in eukaryotic expression vectors, e.g., SV40 CMB vectors. Other exemplary eukaryotic vectors include pMSG, pAV009/A+, pMTO10/A+, pMAMneo-5, and any other vector allowing expression of proteins under the direction of such promoters as the SV40 early promoter, SV40 later promoter, metallothionein promoter, human cytomegalovirus promoter, murine mammary tumor virus promoter, Rous sarcoma virus promoter, polyhedrin promoter, or other promoters shown effective for expression in eukaryotic cells.

Therapeutic quantities of plasmid DNA can be produced for example, by fermentation in *E. coli*, followed by purification. Aliquots from the working cell bank are used to inoculate growth medium, and grown to saturation in shaker flasks or a bioreactor according to well known techniques. Plasmid DNA can be purified using standard bioseparation technologies such as solid phase anion-exchange resins. If required, supercoiled DNA can be isolated from the open circular and linear forms using gel electrophoresis or other methods.

Purified plasmid DNA can be prepared for injection using a variety of formulations. The simplest of these is reconstitution of lyophilized DNA in sterile phosphate-buffer saline (PBS). This approach, known as "naked DNA," is particularly suitable for intramuscular (IM) or intradermal (ID) administration.

To maximize the immunotherapeutic effects of minigene DNA vaccines, alternative methods for formulating purified plasmid DNA may be desirable. A variety of methods have been described, and new techniques may become available. Cationic lipids can also be used in the formulation (see, e.g., as described by WO 93/24640; Mannino & Gould-Fogerite, *BioTechniques* 6(7): 682 (1988); U.S. Pat No. 5,279,833; WO 91/06309; and Felgner, et al., *Proc. Nat'l Acad. Sci. USA* 84:7413 (1987). In addition, glycolipids, fusogenic liposomes, peptides and compounds referred to collectively as protective, interactive, non-condensing compounds (PINC) could also be complexed to purified plasmid DNA to influence variables such as stability, intramuscular dispersion, or trafficking to specific organs or cell types.

Selection of an HIV specific epitope.

Highly antigenic proteins or epitopes for provoking an immune response selective for a specific retroviral pathogen are known. Typically, HIV is the

target retroviral pathogen. With minor exceptions, the following discussion of HIV epitopes is applicable to other retroviruses except for the differences in sizes of the respective viral proteins. Nucleic acids for inclusion in the expression constructs can include sequences encoding either structural or non-structural proteins or epitopes corresponding to regions of the proteins. The envelope, gag, and protease genes are preferred proteins or sources of epitopes for inclusion in the nucleic acid expression vector, but other proteins can also be used. Non-structural genes include the *rev*, *tat*, *nef*, *vif*, and *vpr* genes and these may also be included as components of the nucleic acid vaccines used in the invention.

Characterization of the Immune Response in Vaccinated Individuals

The vaccine regimen can be delivered to individuals at risk for infection with HIV or to patients who are infected with the virus. In order to assess the efficacy of the vaccine, the immune response can be assessed by measuring the induction of $CD4^+$, $CD8^+$, and antibody responses to particular epitopes. Moreover, viral titer can be measured in patients treated with the vaccine who are already infected. These parameters can be measured using techniques well known to those of skill in the art. Examples of such techniques are described below.

$CD4^+$ T cell counts

To assess the effectiveness of the vaccine combination in a recipient and to monitor the immune system of a patient already infected with the virus who is a candidate for treatment with the vaccine regimen, it is important to measure $CD4^+$ T cell counts. A detailed description of this procedure was published by Janet K.A.

Nicholson, Ph.D *et al.* 1997 Revised Guidelines for Performing $CD4^+$ T-Cell Determinations in Persons Infected with Human Immunodeficiency Virus (HIV) in The Morbidity and Mortality Weekly Report, 46(RR-2):[inclusive page numbers], Feb 14, 1997. Centers for Disease Control.

In brief, most laboratories measure absolute $CD4^+$ T-cell levels in whole blood by a multi-platform, three-stage process. The $CD4^+$ T-cell number is the product of three laboratory techniques: the white blood cell (WBC) count; the percentage of WBCs that are lymphocytes (differential); and the percentage of lymphocytes that are $CD4^+$ T-cells. The last stage in the process of measuring the

percentage of CD4⁺ T-lymphocytes in the whole-blood sample is referred to as "immunophenotyping by flow cytometry.

Immunophenotyping refers to the detection of antigenic determinants (which are unique to particular cell types) on the surface of WBCs using antigen-specific monoclonal antibodies that have been labeled with a fluorescent dye or fluorochrome (e.g., phycoerythrin [PE] or fluorescein isothiocyanate [FITC]). The fluorochrome-labeled cells are analyzed by using a flow cytometer, which categorizes individual cells according to size, granularity, fluorochrome, and intensity of fluorescence. Size and granularity, detected by light scattering, characterize the types of WBCs (i.e., granulocytes, monocytes, and lymphocytes). Fluorochrome-labeled antibodies distinguish populations and subpopulations of WBCs.

Systems for measuring CD4⁺ cells are commercially available. For example Becton Dickinson's FACSCount System automatically measure absolutes CD4⁺, CD8⁺, and CD3⁺ T lymphocytes. It is a self-contained system, incorporating instrument, reagents, and controls.

A successful increase of CD4⁺ cell counts would be a 2X or higher increase in the number of CD4⁺ cells.

Measurements of CD8⁺ Responses

CD8⁺ T-cell responses may be measured, for example, by using tetramer staining of fresh or cultured PBMC, ELISPOT assays or by using functional cytotoxicity assays, which are well-known to those of skill in the art. For example, a functional cytotoxicity assay can be performed as follows. Briefly, peripheral blood lymphocytes from patients are cultured with HIV peptide epitope at a density of about five million cells/ml. Following three days of culture, the medium is supplemented with human IL-2 at 20 units/ml and the cultures are maintained for four additional days. PBLs are centrifuged over Ficoll-Hypaque and assessed as effector cells in a standard ⁵¹Cr-release assay using U-bottomed microtiter plates containing about 10⁴ target cells with varying effector cell concentrations. All cells are assayed twice.

Autologous B lymphoblastoid cell lines are used as target cells and are loaded with peptide by incubation overnight during ⁵¹Cr labeling. Specific release is calculated in the following manner: (experimental release-spontaneous release)/(maximum release-spontaneous release) x 100. Spontaneous release is generally less than 20% of

maximal release with detergent (2% Triton X-100) in all assays. A successful CD8⁺ response occurs when the induced cytolytic activity is above 10% of controls.

Another measure of CD8⁺ responses provides direct quantification of antigen-specific T cells by staining with Fluorescein-labeled HLA tetrameric complexes (Altman, J. D. *et al.*, *Proc. Natl. Acad. Sci. USA* 90:10330, 1993; Altman, J. D. *et al.*, *Science* 274:94, 1996). Other assays include staining for intracellular lymphokines, and γ -interferon release assays or ELISPOT assays. Tetramer staining, intracellular lymphokine staining and ELISPOT assays all are sensitive measures of T cell response (Lalvani, A. *et al.*, *J. Exp. Med.* 186:859, 1997; Dunbar, P. R. *et al.*, *Curr. Biol.* 8:413, 1998; Murali-Krishna, K. *et al.*, *Immunity* 8:177, 1998).

Viral titer

There are a variety of ways to measure viral titer in a patient. A review of the state of this art can be found in the Report of the NIH To Define Principles of Therapy of HIV Infection as published in the ;Morbidity and Mortality Weekly Reports, April 24, 1998, Vol 47, No. RR-5, Revised 6/17/98. It is known that HIV replication rates in infected persons can be accurately gauged by measurement of plasma HIV concentrations.

HIV RNA in plasma is contained within circulating virus particles or virions, with each virion containing two copies of HIV genomic RNA. Plasma HIV RNA concentrations can be quantified by either target amplification methods (e.g., quantitative RT polymerase chain reaction [RT-PCR], Amplicor HIV Monitor assay, Roche Molecular Systems; or nucleic acid sequence-based amplification, [NASBA[®]], NucliSens[™] HIV-1 QT assay, Organon Teknika) or signal amplification methods (e.g., branched DNA [bDNA], Quantiplex[™] HIV RNA bDNA assay, Chiron Diagnostics). The bDNA signal amplification method amplifies the signal obtained from a captured HIV RNA target by using sequential oligonucleotide hybridization steps, whereas the RT-PCR and NASBA[®] assays use enzymatic methods to amplify the target HIV RNA into measurable amounts of nucleic acid product. Target HIV RNA sequences are quantitated by comparison with internal or external reference standards, depending upon the assay used.

Formulation of Vaccines and Administration

The administration procedure for recombinant virus or DNA is not critical. Vaccine compositions (*e.g.*, compositions containing the poxvirus recombinants or DNA) can be formulated in accordance with standard techniques well known to those skilled in the pharmaceutical art. Such compositions can be administered in dosages and by techniques well known to those skilled in the medical arts taking into consideration such factors as the age, sex, weight, and condition of the particular patient, and the route of administration.

The vaccines can be administered prophylactically or therapeutically. In prophylactic administration, the vaccines are administered in an amount sufficient to induce CD8⁺ and CD4⁺, or antibody, responses. In therapeutic applications, the vaccines are administered to a patient in an amount sufficient to elicit a therapeutic effect, *i.e.*, a CD8⁺, CD4⁺, and/or antibody response to the HIV-1 antigens or epitopes encoded by the vaccines that cures or at least partially arrests or slows symptoms and/or complications of HIV infection. An amount adequate to accomplish this is defined as "therapeutically effective dose." Amounts effective for this use will depend on, *e.g.*, the particular composition of the vaccine regimen administered, the manner of administration, the stage and severity of the disease, the general state of health of the patient, and the judgment of the prescribing physician.

The vaccine can be administered in any combination, the order is not critical. In some instances, for example, a DNA HIV vaccine is administered to a patient more than once followed by delivery of one or more administrations of the recombinant pox virus vaccine. The recombinant viruses are typically administered in an amount of about 10⁴ to about 10⁹ pfu per inoculation; often about 10⁴ pfu to about 10⁶ pfu. Higher dosages such as about 10⁴ pfu to about 10¹⁰ pfu, *e.g.*, about 10⁵ pfu to about 10⁹ pfu, or about 10⁶ pfu to about 10⁸ pfu, can also be employed. For example, a NYVAC-HIV vaccine can be inoculated by the intramuscular route at a dose of about 10⁸ pfu per inoculation, for a patient of 170 pounds.

Suitable quantities of DNA vaccine, *e.g.*, plasmid or naked DNA can be about 1 µg to about 100 mg, preferably 0.1 to 10 mg, but lower levels such as 0.1 to 2 mg or 1-10 µg can be employed. For example, an HIV DNA vaccine, *e.g.*, naked DNA or polynucleotide in an aqueous carrier, can be injected into tissue, *e.g.*, intramuscularly or intradermally, in amounts of from 10 µl per site to about 1 ml per

site. The concentration of polynucleotide in the formulation is from about 0.1 µg/ml to about 20 mg/ml.

The vaccines may be delivered in a physiologically compatible solution such as sterile PBS in a volume of, *e.g.*, one ml. The vaccines can also be lyophilized prior to delivery. As well known to those in the art, the dose may be proportional to weight.

The compositions included in the vaccine regimen of the invention can be co-administered or sequentially administered with other immunological, antigenic or vaccine or therapeutic compositions, including an adjuvant, a chemical or biological agent given in combination with or recombinantly fused to an antigen to enhance immunogenicity of the antigen. Additional therapeutic products can include, *e.g.*, interleukin-2 (IL-2) or CD40 ligand in an amount that is sufficient to further potentiate the CD8⁺ and CD4⁺ T-cell responses. Such other compositions can also include purified antigens from the immunodeficiency virus or from the expression of such antigens by a second recombinant vector system which is able to produce additional therapeutic compositions. For examples, these compositions can include a recombinant poxvirus which expresses other immunodeficiency antigens or biological response modifiers (*e.g.*, cytokines or co-stimulating molecules). Examples of adjuvants which also may be employed include Freund's complete adjuvant and MPL-TDM adjuvant (monophosphoryl Lipid A, synthetic trehalose dicorynomycolate). Again, co-administration is performed by taking into consideration such known factors as the age, sex, weight, and condition of the particular patient, and, the route of administration.

The viral and DNA vaccines can additionally be complexed with other components such as lipids, peptides, polypeptides and carbohydrates for delivery.

The DNA vaccines are administered by methods well known in the art as described in Donnelly *et al.* (*Ann. Rev. Immunol.* 15:617-648 (1997)); Felgner *et al.* (U.S. Patent No. 5,580,859, issued December 3, 1996); Felgner (U.S. Patent No. 5,703,055, issued December 30, 1997); and Carson *et al.* (U.S. Patent No. 5,679,647, issued October 21, 1997). The vectors can also be complexed to particles or beads that can be administered to an individual, for example, using a vaccine gun. One skilled in the art would know that the choice of a pharmaceutically acceptable carrier,

including a physiologically acceptable compound, depends, for example, on the route of administration of the expression vector.

Vaccines may be delivered via a variety of routes. Typical delivery routes include parenteral administration, *e.g.*, intradermal, intramuscular or subcutaneous delivery. Other routes include oral administration, intranasal, and intravaginal routes. For DNA vaccines in particular, the vaccines can be delivered to the interstitial spaces of tissues of an individual (Felgner *et al.*, U.S. Patent Nos. 5,580,859 and 5,703,055). Administration of DNA vaccines to muscle is also a frequently used method of administration, as is intradermal and subcutaneous injections and transdermal administration. Transdermal administration, such as by iontophoresis, is also an effective method to deliver nucleic acid vaccines to muscle. Epidermal administration of expression vectors of the invention can also be employed. Epidermal administration involves mechanically or chemically irritating the outermost layer of epidermis to stimulate an immune response to the irritant (Carson *et al.*, U.S. Patent No. 5,679,647).

The vaccines can also be formulated for administration via the nasal passages. Formulations suitable for nasal administration, wherein the carrier is a solid, include a coarse powder having a particle size, for example, in the range of about 10 to about 500 microns which is administered in the manner in which snuff is taken, *i.e.*, by rapid inhalation through the nasal passage from a container of the powder held close up to the nose. Suitable formulations wherein the carrier is a liquid for administration as, for example, nasal spray, nasal drops, or by aerosol administration by nebulizer, include aqueous or oily solutions of the active ingredient. For further discussions of nasal administration of AIDS-related vaccines, references are made to the following patents, US 5,846,978, 5,663,169, 5,578,597, 5,502,060, 5,476,874, 5,413,999, 5,308,854, 5,192,668, and 5,187,074.

Examples of vaccine compositions of use for the invention include liquid preparations, for orifice, *e.g.*, oral, nasal, anal, vaginal, etc. administration, such as suspensions, syrups or elixirs; and, preparations for parenteral, subcutaneous, intradermal, intramuscular or intravenous administration (*e.g.*, injectable administration) such as sterile suspensions or emulsions. In such compositions the recombinant poxvirus, expression product, immunogen, DNA, or modified gp120 or gp160 may be in admixture with a suitable carrier, diluent, or excipient such as sterile water, physiological saline, glucose or the like.

The vaccines can be incorporated, if desired, into liposomes, microspheres or other polymer matrices (Felgner *et al.*, U.S. Patent No. 5,703,055; Gregoriadis, *Liposome Technology*, Vols. I to III (2nd ed. 1993), each of which is incorporated herein by reference). Liposomes, for example, which consist of
5 phospholipids or other lipids, are nontoxic, physiologically acceptable and metabolizable carriers that are relatively simple to make and administer.

Liposome carriers may serve to target a particular tissue or infected cells, as well as increase the half-life of the vaccine. Liposomes include emulsions, foams, micelles, insoluble monolayers, liquid crystals, phospholipid dispersions,
10 lamellar layers and the like. In these preparations the vaccine to be delivered is incorporated as part of a liposome, alone or in conjunction with a molecule which binds to, *e.g.*, a receptor prevalent among lymphoid cells, such as monoclonal antibodies which bind to the CD45 antigen, or with other therapeutic or immunogenic compositions. Thus, liposomes either filled or decorated with a desired immunogen
15 of the invention can be directed to the site of lymphoid cells, where the liposomes then deliver the immunogen(s). Liposomes for use in the invention are formed from standard vesicle-forming lipids, which generally include neutral and negatively charged phospholipids and a sterol, such as cholesterol. The selection of lipids is generally guided by consideration of, *e.g.*, liposome size, acid lability and stability of
20 the liposomes in the blood stream. A variety of methods are available for preparing liposomes, as described in, *e.g.*, Szoka, *et al.*, *Ann. Rev. Biophys. Bioeng.* 9:467 (1980), U.S. Patent Nos. 4,235,871, 4,501,728, 4,837,028, and 5,019,369.

All publications and patent applications cited in this specification are herein incorporated by reference as if each individual publication or patent application
25 were specifically and individually indicated to be incorporated by reference.

Although the foregoing invention has been described in some detail by way of illustration and example for purposes of clarity of understanding, it will be readily apparent to those of ordinary skill in the art in light of the teachings of this invention that certain changes and modifications may be made thereto without departing
30 from the spirit or scope of the appended claims.

EXAMPLES

The following examples are provided by way of illustration only and not by way of limitation. Those of skill will readily recognize a variety of noncritical parameters which could be changed or modified to yield essentially similar results.

Example 1. Administration of DNA priming vaccines in combination with NYVAC-SIV_{gag-pol-env} in Rhesus macaques

The study design included 24 animals which were divided into three groups, A, B, and C as follows:

Group A: eight animals vaccinated with the nonrecombinant NYVAC control

Group B: eight animals vaccinated with NYVAC-SIV_{gag-pol-env}

Group C: eight animals vaccinated with 3 DNA immunizations with 2 constructs expressing the Gag and Env proteins of SIV₂₃₉, followed by inoculation NYVAC-SIV_{gag-pol-env} at the indicated time.

In each group, macaques carrying the MHC class I molecule MAMU-A*01 were included to quantitate the CD3⁺CD8⁺ T-cell immune response in the blood. Animals were immunized with either 4 inoculation of 10⁸ pfu of NYVAC or NYVAC-SIV or with 3 inoculations of DNA (4 mg intramuscularly and 1 mg subcutaneously of each plasmid) followed by two inoculation of NYVAC-SIV at the indicated times (Fig. 1).

The following measure of immune response were obtained:

(1) *in vitro* lymphoproliferative responses to gp120 and p27 Gag

(2) *ex vivo* percentage of CD3⁺CD8⁺ T-cells staining the p11C_{C→M}-Mamu-A*01 tetramer in peripheral blood monocytes.

(3) expansion of the CD3⁺CD8⁺ tetramer-positive population *in vitro* in the presence of a specific peptide (p11C, C→M)

(4) ELISPOT for γ -IFN secretion following stimulation of CD8⁺ T-cells with virus-specific nonamers

(5) Serum Ab response to the Gag and gp120 env protein antigens can also be measured.

The results showed that low levels of lymphoproliferative responses to p27 Gag and gp120 were observed in animals in group B (Fig. 2) immunized with

NYVAC-SIV_{gag-pol-env} alone. A marked lymphoproliferative response was observed in Group C, however. These animals received 3 inoculations of DNA prior to vaccination with NYVAC-SIV_{gag-pol-env}. High lymphoproliferative responses to p27 Gag and Env occurred in seven of the eight animals (Fig. 2) and overall, a difference of approximately ten-fold was observed in comparison to group B animals. Additionally, in all of the MAMU-A*01 animals, expansion of the *ex vivo* and cultured tetramer-positive cells from the blood was observed.

To further assess whether DNA priming resulted in potentiation and an increase in breadth of the immune response, ELISPOT analysis of γ -INF-producing cells following a specific peptide stimulation was performed. The results are shown in Fig. 3. The peptides used to stimulate the responses are shown in the X-axes of the middle and bottom panels. An asterisk above the bar (top panel) indicates values obtained by assaying frozen cells (in control experiments, cell freezing decreased the number of peptide-specific spots by 0-20%). Controls include NYVAC-SIV-*gpe*-vaccinated, MAMU A*01-negative animal 17M, mock NYVAC-vaccinated, MAMU A*01-negative animal 11 M, and mock NYVAC-vaccinated, MAMU A*01-positive animal 671.

The combination of DNA vaccination with NYVAC-SIV vaccination expanded the immunodominant response (p181) in all animals in group C more than 10-fold compared to the animals in group B (Fig. 3, upper panel). These responses were not only of a greater magnitude, but also of longer duration. Moreover, the animals in group C responded to more SIV epitopes and, again, the responses were higher at 2 weeks following immunization (Fig. 3, lower panels).

Gag181-specific tetramer staining of fresh PBMC at week 53 and week 76 was also performed. The results (Fig. 4) showed that DNA vaccination also increased the frequency of memory T-cells recognizing the p11C, C→M tetramer, as exemplified by the detection of a clear population of these CD8⁺ T-cells in the blood of five of five animals of group C and one of four in group B at week 76.

Similarly, the functional activity of these cells in cytolytic assays indicated that five of five macaques in group C had CTL against viral epitopes whereas only two of five in group B did (Fig. 5). The data in Fig. 5 show T-cell responses to various SIV epitopes measured using the ELISPOT and ⁵¹Cr-release assay. The bar charts represent the results of an IFN- γ ELISPOT assays with a

specific MAMU A*01-restricted peptide indicated for each set of bars at the indicated times. The values exceeding the chart scale are indicated by number at the top of the bar. Asterisks indicate the values obtained using frozen cells; all other assays were performed using fresh PBMC. "Ctrl" indicates unrelated control peptide; "N.D." indicates not done. Line charts represent the percentage of a specific killing of unpulsed control cells or cells pulsed with a specific MAMU A*01-restricted peptide. All assays were performed using the cells from 7 day cultures with a specific peptide at week 53 or 56, as indicated. "E:T" represents the effector to target cell ratio. The percentage value in the top right corner indicates the percentage of Gag181 tetramer-staining CD3⁺CD8⁺ cells in culture PBMC.

Following intrarectal challenge with the highly pathogenic SIV_{mrc251} (561) strain, most animals became viremic except one of the animals in group C. The ability of vaccinated animals to suppress viremia was assessed within the first 28 days and, as demonstrated in Fig. 6, macaques immunized with DNA at first were better able to control viremia than control macaques. Interestingly, quantification of the anamnestic response in vaccinated animals using the Gag 181 tetramer indicated that in DNA primed animals, the response was higher and sustained (Fig. 7).

Because it has been previously demonstrated that Mamu-A*01-positive animals are genetically advantaged and better control viremia than Mamu-A*01-negative animals, virus load and anamnestic response were investigated independently in Mamu-A*01-positive and -negative macaques. As demonstrated in Fig. 8, analysis of virus load in Mamu-A*01-positive control animals (including data from historical control animals challenged with the same virus stock by the same route to increase the statistical power of our analyses) and vaccinated animals in group C demonstrated a vaccine effect, and a similar effect was observed in Mamu-A*01-negative macaques (bottom panel). Thus, DNA vaccination ameliorated the virological outcome even in animals with an inherent genetic predisposition to control viremia.

Data in the literature indicated that DNA priming followed by MVA boost was not so effective in reducing virus load (*see, e.g., Hanke et al., J. Virol.* 73:7524-7532, 1999). The demonstration that DNA in combination with NYVAC significantly improved viral outcome was surprising and may be dependent on inherent features of this poxvirus vector.

These data demonstrate that DNA vaccination greatly potentiates and increases the breadth of the immune response induced by a NYVAC-based vaccine and shows that this vaccine combination increases the immunogenicity and efficacy of the highly attenuated poxvirus vectors.

5 ALVAC-based vaccine are similarly analyzed demonstrating that they also potentiate the immune response when used in conjunction with DNA vaccines.

Example 2. Administration to a person

10 A vaccine regimen of a DNA priming vaccine followed by inoculation with a vaccine such as NYVAC or ALVAC, is used prophylactically in individuals at risk for HIV infection. (Such a vaccine regimen can also be used therapeutically for HIV-infected patients).

15 The individual is injected with a DNA priming vaccine that, *e.g.*, expresses the HIV-1 *gag*, *pro*, *tat*, *nef*, *rev*, and *env* genes. Multiple priming inoculations are typically administered. The amount of DNA administered is typically 800 µg intramuscularly or 200 µg intradermally. After an interval determined by the physician, the patient is subsequently injected with a vaccine comprising about 10^8 pfu of a recombinant pox virus, *e.g.* NYVAC, expressing HIV-1 *gag*, *pro*, *tat*, *nef*, *rev*, and *env* epitopes.

20 The patient's immune response is evaluated ($CD4^+$ proliferative response, cytotoxic $CD8^+$ T-cell activity, *etc.*) and a decision is made as to whether and when to immunize again.

The combination of administration of the DNA vaccine followed by immunization with the recombinant NYVAC vaccine provides a protective immune response in uninfected patients and a therapeutic effect in those individuals already infected with HIV-

WHAT IS CLAIMED IS:

1. A method of potentiating a $CD8^+$ response to human immunodeficiency virus-1 (HIV-1) epitopes in a human by administering a combination of vaccines, said method comprising:

administering a nucleic acid vaccine;

administering a recombinant pox virus vaccine encoding one or more of the same antigens encoded by the nucleic acid vaccine;

wherein the nucleic acid and the recombinant pox virus vaccines enter the cells of the human and intracellularly produce HIV-specific peptides that are presented on the cell's MHC class I molecules in an amount sufficient to stimulate a $CD8^+$ response, and further, wherein administration of the combination of vaccines potentiates the immune response compared to administration of either the nucleic acid or the recombinant pox virus vaccine by itself.

2. A method of claim 1 wherein the vaccine is an attenuated recombinant pox virus vaccine.

3. A method of claim 2 wherein the attenuated recombinant pox virus vaccine is selected from the group consisting of NYVAC and ALVAC.

4. A method of claim 1, wherein the nucleic acid vaccine is a DNA vaccine.

5. A method of claim 1 wherein the HIV-specific peptides are structural viral peptides

6. A method of claim 1 wherein the HIV-specific peptides are non-structural viral peptides.

7. A method of claim 1 wherein the vaccines further comprise an adjuvant.

8. A method of claim 1 further comprising two administrations of nucleic acid vaccine.

9. A method of claim 8 comprising three administrations of the nucleic acid vaccine.

5 10. A method of claim 1, wherein the nucleic acid vaccine is administered before the recombinant pox virus vaccine.

11. A method of claim 1, wherein the human is infected with HIV-1.

10 12. A method of claim 11, wherein the human has a viral load of less than 10,000 copies per milliliter.

13. A method of claim 1, wherein the human is not infected with HIV-1.

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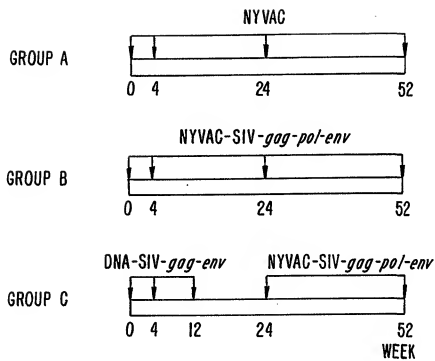


FIG. 1.

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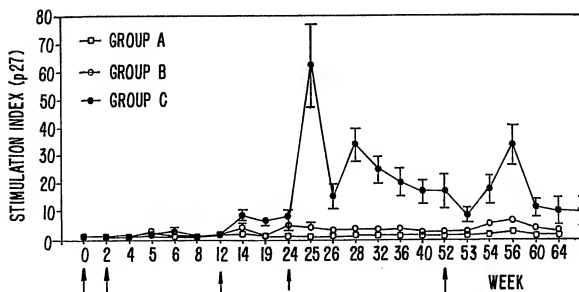
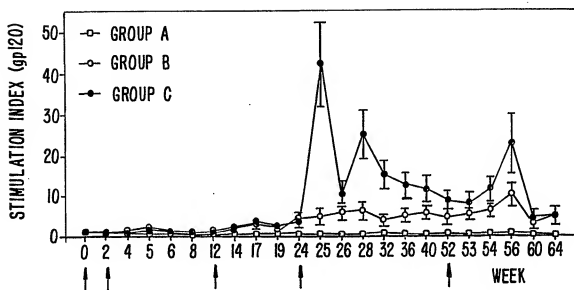
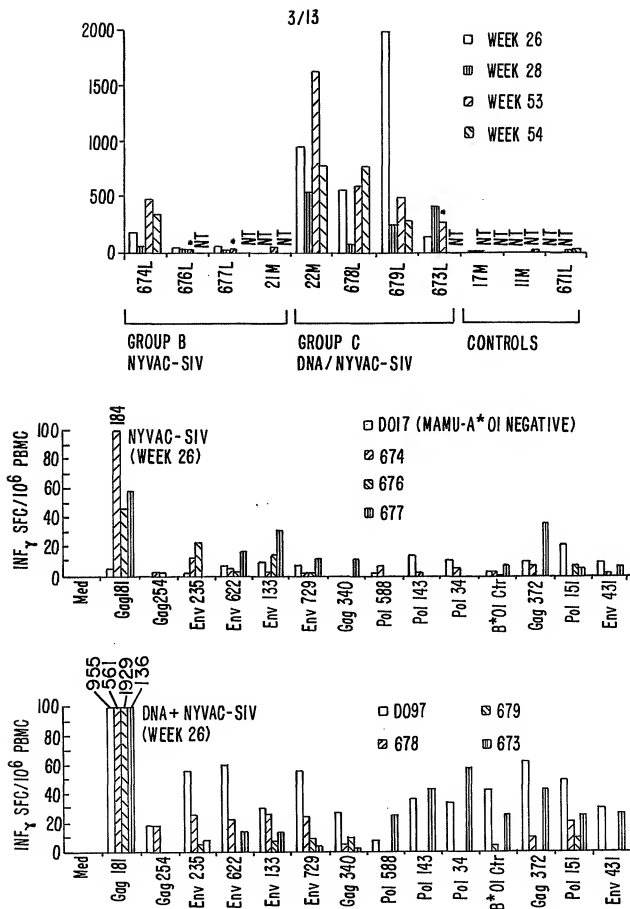


FIG 2



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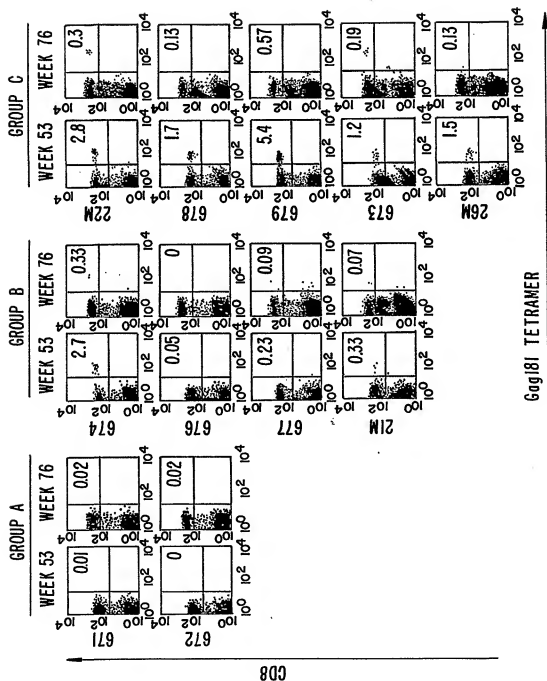


FIG. 4.

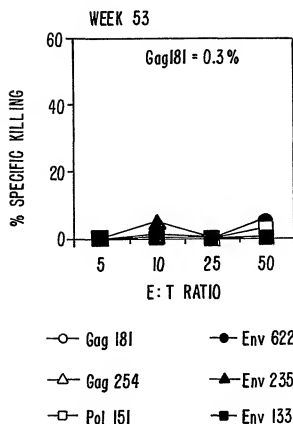
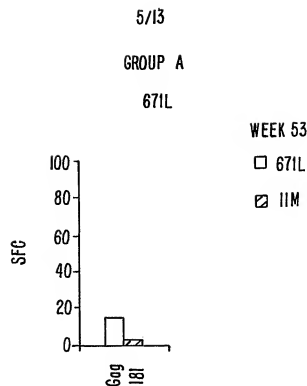


FIG. 5A.

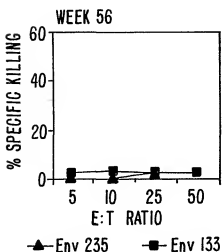
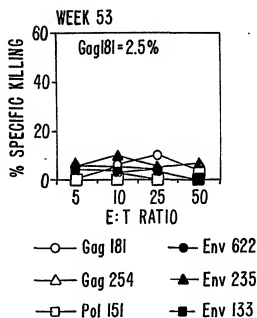
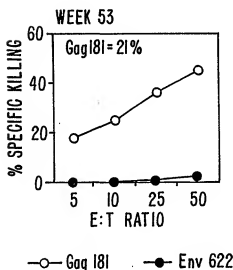
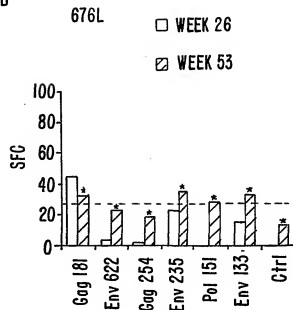
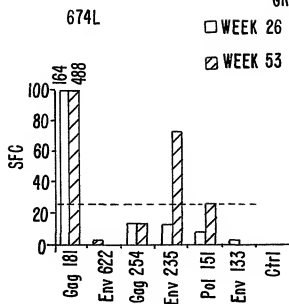
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GROUP B

FIG. 5B-1.

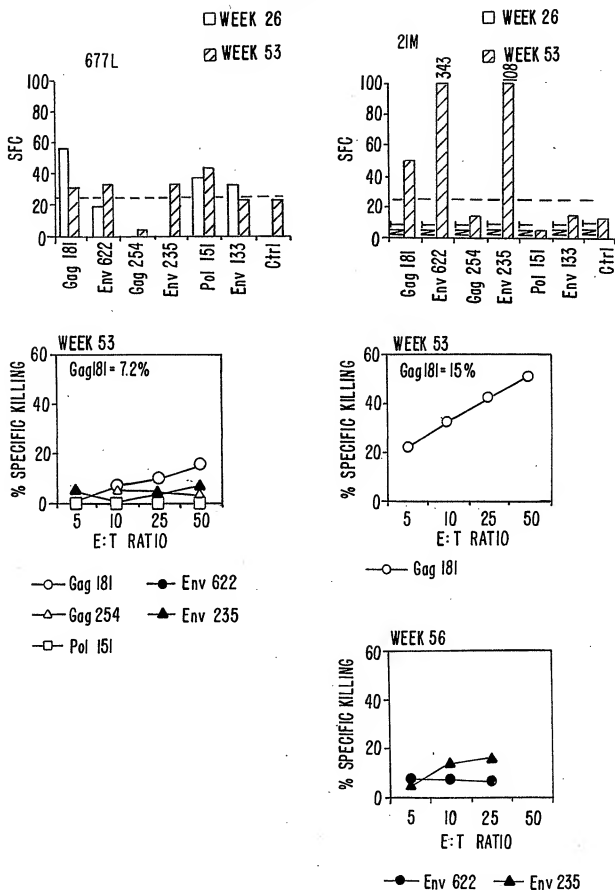
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GROUP B

FIG. 5B-2.

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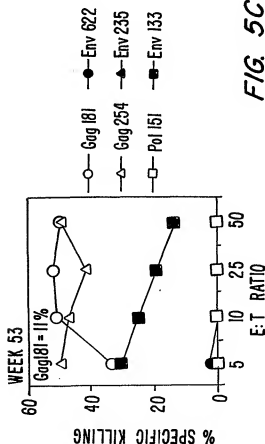
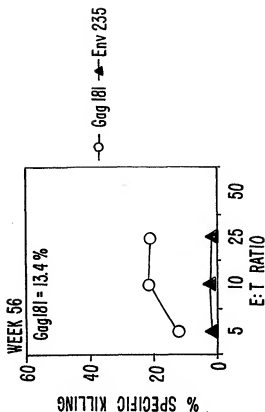
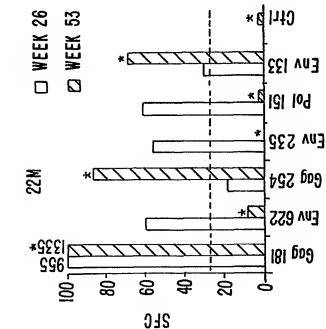
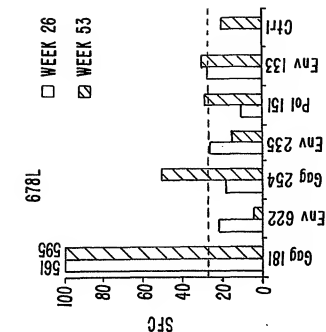


FIG. 5C-1.

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GROUP C

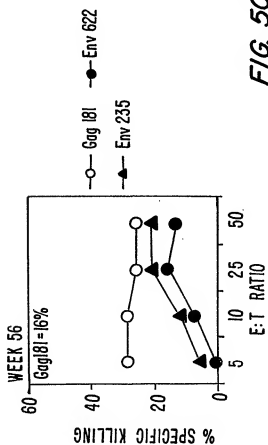
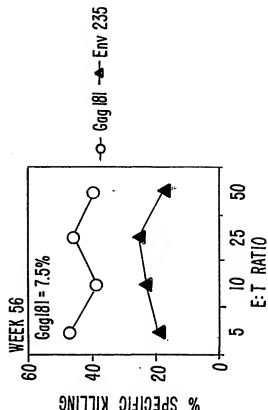
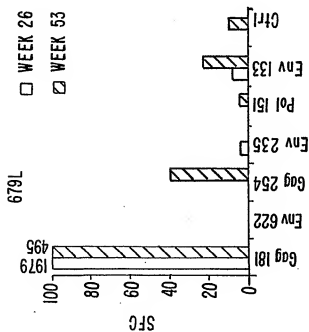
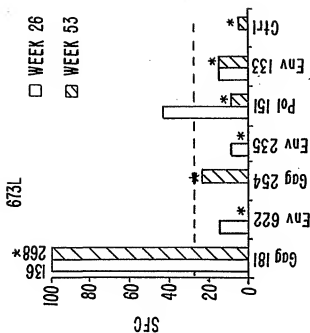


FIG. 5C-2.

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GROUP C

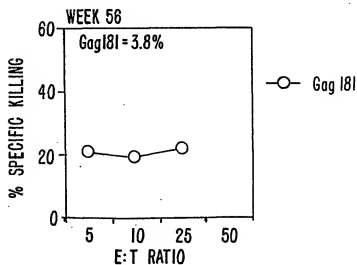
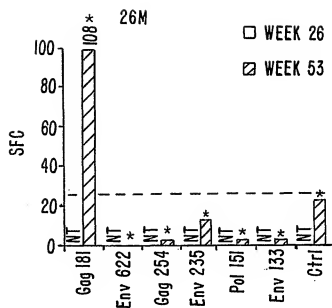
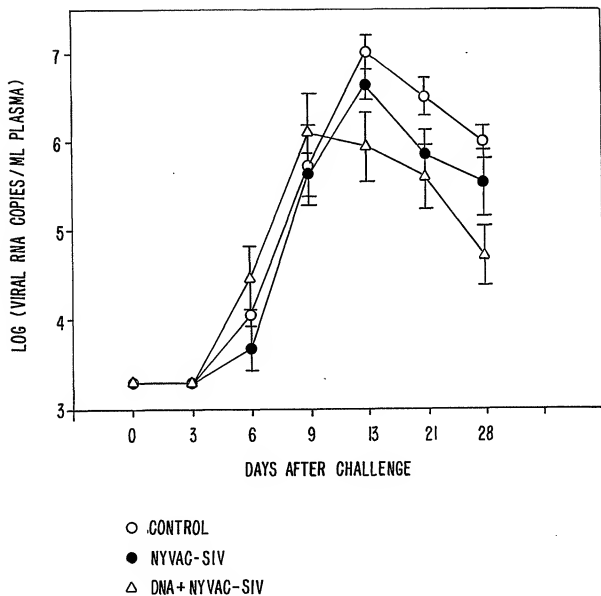
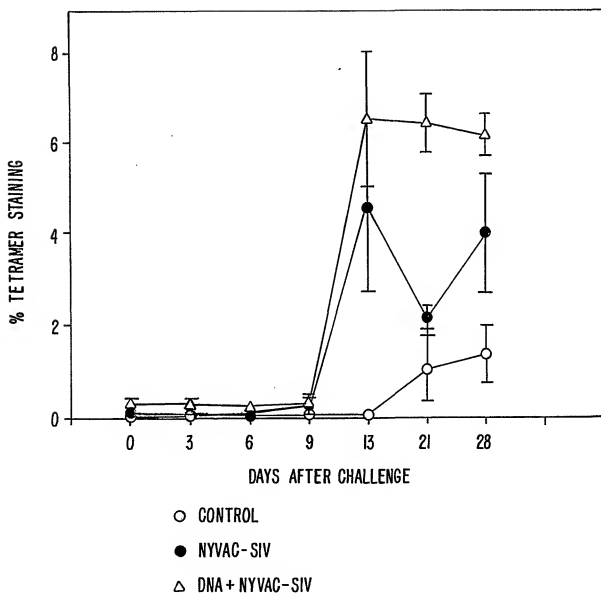


FIG. 5C-3.

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**FIG. 6.**

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**FIG. 7.**

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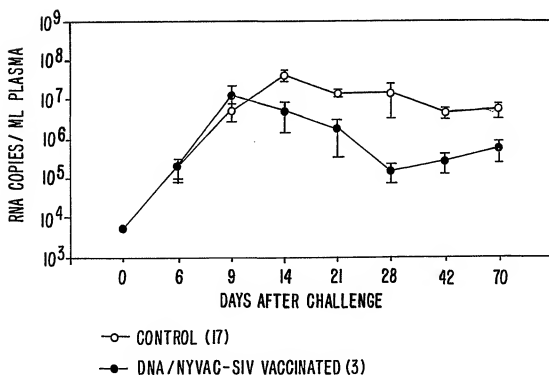
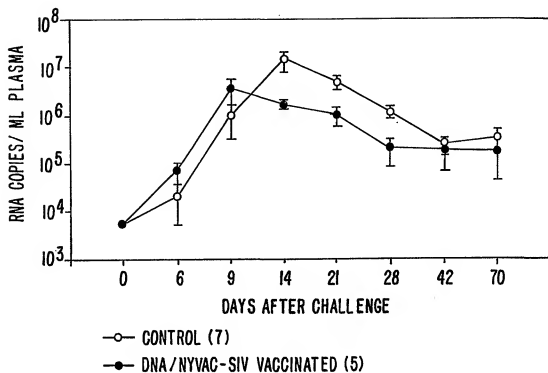


FIG. 8.

INTERNATIONAL SEARCH REPORT

International Application No.

PCT/US 01/13968

A. CLASSIFICATION OF SUBJECT MATTER

IPC 7 A61K39/21 A61P31/18

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC 7 A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

EMBASE, EPO-Internal, BIOSIS, MEDLINE, CHEM ABS Data, SCISEARCH, WPI Data

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 98 56919 A (GILBERT SARAH CATHERINE ;HILL ADRIAN VIVIAN SINTON (GB); PLEBANSKI) 17 December 1998 (1998-12-17) the whole document ---	1-13
X	RAMSHAW I.A. ET AL: "The prime - boost strategy: Exciting prospects for improved vaccination." IMMUNOLOGY TODAY, (2000) 21/4 (163-165). , - April 2000 (2000-04) XP002179214 the whole document --- -/-	1-13



Further documents are listed in the continuation of box C.



Patent family members are listed in annex.

* Special categories of cited documents:

- *A* document defining the general state of the art which is not considered to be of particular relevance
- *E* earlier document but published on or after the international filing date
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Date of the actual completion of the international search

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Niemann, F

INTERNATIONAL SEARCH REPORT

International Application No.

rCT/US 01/13968

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
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X	<p>HANKE T ET AL: "Effective induction of HIV-specific CTL by multi-epitope using gene gun in a combined vaccination regime" VACCINE, BUTTERWORTH SCIENTIFIC. GUILDFORD, GB, vol. 17, no. 6, February 1999 (1999-02), pages 589-596, XP004153817 ISSN: 0264-410X the whole document</p> <p>---</p>	1-13
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INTERNATIONAL SEARCH REPORT

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C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

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Information on patent family members

International Application No

rCT/US 01/13968

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